

Amendments to the Specification

Please replace the paragraph on page 1, lines 18-28 with the following paragraph.

--The zinc finger protein family encompasses a broad variety of proteins with assorted functions. One relatively uncommon class of zinc finger proteins contains fingers of the Cys-Cys-Cys-His (SEQ ID NO:48) (CCCH) type, in which three cysteines and one histidine are thought to coordinate a single atom of zinc. Members of a very small subclass of the larger family of CCCH zinc finger proteins contain two tandem zinc fingers consisting of Cx₈Cx₅Cx₃H (SEQ ID NO:47) (wherein "x" refers to variable amino acids), spaced exactly 18 amino acids apart. The prototype of proteins of this CCCH double zinc finger subclass is tristetraprolin (TTP), also known as TIS11 and Nup475. TTP is localized to the nucleus of quiescent fibroblasts, but is rapidly phosphorylated on serine residues and translocated to the cytosol after stimulation with serum or other mitogens. TTP is almost completely cytosolic in macrophages.--

Please replace the table bridging pages 10 to 12, with the following table.

aa1-aa7:	RYKTELC (<u>SEQ ID NO: 42</u>)
aa8:	R/s
aa9:	P/T/r
aa10:	F/Y
aa11:	E/S/a
aa12:	E
aa13:	S/N/t/s
aa14:	G
aa15:	A/R/T/S/f
aa16:	C
aa17:	K/R/a
aa18:	Y
aa19:	G/a/r

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aa20: E/A/D/n

aa21: K/R

aa22-aa27: CQFAHG (SEQ ID NO: 43)

aa28: L/F/I/p/k

aa29: H/G/i/s

aa30: E/D

aa31: L

aa32: R/H

aa33: S/Q/v/e/p

aa34: L/A/P

aa35: T/N/s/v

aa36: R/q

aa37-aa45: HPKYKTELC (SEQ ID NO: 44)

aa46: R/H

aa47: T/K/s

aa48: F/Y

aa49: H/Y

aa50: T/L/n/v

aa51: I/Q/a/l/y

aa52: G

aa53: F/R/y/e/t

aa54: C

aa55: P/v/n

aa56: Y

aa57: G/v
aa58: P/S/t/l
aa59-aa60: RC
aa61: H/l/n
aa62: F
aa63: I/v
aa64: H

Please replace the paragraph bridging page 15 and 16 with the following paragraph.

-- Two other polypeptides bearing structural similarity to TTP of this subclass have been identified to date in mammals: cMG1 (TIS11b, ERF1, Berg-36) (Barnard et al., Nucleic Acids Res 21:3580, 1993; Gomperts et al., Oncogene 5:1081-1083, 1990; Ning et al., Biochem Soc Trans 25:306S, 1997; and Varnum et al., Mol Cell Biol 11:1754-1758, 1991); and TIS11d (ERF2) (Varnum et al, supra; and Nie et al., Gene 152:285-286, 1995). Like TTP, ERF1/TIS11b and ERF2/TIS11d contain the two typical CCCH fingers, spaced 18 amino acids apart, with the sequence RYKTEL (SEQ ID NO:26) or a variant leading into each finger. Proteins with nearly identical double zinc fingers spaced 18 amino acids apart also have been identified in Drosophila and yeast (Ma et al., Oncogene 9:3329-3334, 1994; Ma and Herschman, Oncogene 10:487-494, 1995; and Thompson et al., Gene 174:225-233, 1996).--

Please replace the paragraph on page 16, lines 11-17 with the following paragraph.

--In addition to the Xenopus homologues (see Fig. 5B) of the three mammalian proteins described above, which all contain two tandem zinc fingers (TZFs), also known is a fourth Xenopus homologue (XC3H-4) containing two CCCH zinc fingers spaced 18 amino acids apart and preceded by the R(K)YKTEL sequence (SEQ ID NO:27), as well as an additional more carboxyl-terminal pair of CCCH zinc fingers that are more closely spaced and lack the lead-in R(K)YKTEL sequence (SEQ ID NO:27) (De et al., Gene 228:133-145, 1999).--

Please replace the paragraph on page 21, lines 1-12 with the following paragraph.

--As shown herein, members of the ERF1 and ERF2 families of TTP-like proteins also stimulate degradation of TNF- α and GM-CSF mRNA. ERF1 and ERF2 are TTP-like proteins of the CCCH double zinc finger class. There is weak similarity between TTP and ERF1 or ERF2 in the non-zinc finger domains, but they are very similar (highly conserved) in the zinc finger domains, both having a lead-in sequence of R(K)YKTEL (SEQ ID NO:27), and then two zinc fingers, spaced 18 amino acids apart, that each have the composition C_xG_xC_xYG_x(K/R)C_xF_xH (SEQ ID NO: 46), where x represents various amino acids. The cloning of ERF1 and the alignment of human and rat ERF1 are described in Barnard et al., *Nucleic Acids Res.* 21:3580, 1993, and the cloning of ERF2 is described in Nie et al., *Gene* 152:285-286, 1995. ERF1 and ERF2 function analogously to TTP, and therefore, competitors and inhibitors of ~~ERF1~~ ERF1 and ERF2 may be identified as described for TTP above.--

Please replace the paragraph on page 30, lines 8-30, with the following paragraph.

A variety of assay methods can be used to determine whether a given compound interferes with TTP or related protein binding to the GM-CSF ARE and the breakdown of GM-CSF mRNA. These would include cell-based experiments, such as the transfection studies in 293 cells cited in Example 3; it can be seen that addition of cell-permeable compounds to the cells that inhibited the TTP-mRNA interaction would result in inhibition of TTP's ability to deadenylate and destroy the mRNA. Such assays could use a variety of more convenient readouts, e.g. luminescent proteins, human growth hormone, chloramphenicol acetyltransferase, beta-galactosidase, etc. Similar cell based studies could also be performed in yeast, where there is considerable precedent for high-throughput screening assays for protein interactions with DNA, RNA and other proteins. Cell-free assays would probably be the most convenient to set up; these would involve extracts from cells expressing TTP or its related proteins (e.g., ERF1, ERF2, etc.) or its active fragments (e.g., the double zinc finger domain), and testing their ability to bind to purified, labeled GM-CSF ARE, assayed by either crosslinking or gel-shift assays as described in the Examples. More conveniently still, these assays could use purified TTP or its

active fragments, or purified members of the TTP-related protein class or their active fragments, or fusion proteins expressing TTP or its related proteins or their fragments. All have been shown to be active at binding and crosslinking to the TNF α ARE. These would use variable lengths of sequence of the ~~GM-CSF~~ GM-CSF ARE – e.g., a probe that corresponds to bases 3390 – 3467 of Genbank accession number X03020, but the experiments with the TNF ARE have shown that this could probably be shortened to a “core” ARE of about 23 bases (bases 1309 to 1332 of Genbank Accession number X02611 and corresponding bases for GM-CSF).

Please replace the paragraph on page 35, lines 4-24, with the following paragraph.

--When indicated, the cells were stimulated with lipopolysaccharide (LPS) (1 μ g/ml) (Sigma) or mouse recombinant TNF α (10 ng/ml) (R & D Systems, Inc., Minneapolis, MN) for different times, and RNA was extracted with the RNeasy kit from Qiagen, Inc. (Valencia, CA), according to the directions provided by the manufacturer. RNA was analyzed by Northern blot as described (25), except that the gels contained 1.5% (w/v) agarose. Filters were sequentially probed with cDNA probes to mouse GM-CSF (plasmid p5'mGM-CSF, containing the sequences of exons I, II, III and part of the exon IV of the mouse GM-CSF (bp 1136-1317, 1415-1456, 2214-2339, 3082-3153 of GenBank accession number X03020), was constructed by RT-PCR using RNA from Raw 264.7 cells treated for 4 hrs. with 1 μ g/ml of LPS as the template for RT. The 5' primer for PCR amplification was 5'gtcgacACTCAGAGAGAAAGGCTAAGG3' (SEQ ID NO:28), and the 3' primer was 5'CATTCAAAGGGgatatcAGTCAG3' (SEQ ID NO:29), where the lower case letters indicate the restriction sites for SalI and EcoRV, respectively (the EcoRV site is a naturally occurring site in the mGM-CSF gene). The resulting PCR product was digested with these enzymes and cloned into the SalI, EcoRV and XbaI sites of the vector pSK- (Stratagene). Correct sequence of the plasmid insert was confirmed by dye terminator cycle sequencing (Perkin-Elmer, Foster City, CA)) and rat GAPDH (26). The 423 bp SalI-EcoRV insert from the GM-CSF and the 1.3 kb EcoRI insert from the GAPDH cDNAs were isolated from low-melting point agarose gels and random primer labeled with α -³²P dCTP for Northern hybridization.--

Please replace the paragraph bridging pages 61 and 62 with the following paragraph.

-- H6E.HGH3' was constructed as follows: a 597 bp NsiI-XbaI fragment in the 3' untranslated region (3' UTR) of H6E that contained five rapid degradation signal sequences was replaced by 110 bp of human growth hormone (HGH) sequence that encode the entire HGH 3' UTR (GenBank accession number M13438). The template used to amplify this fragment was pØGH (Nichols Institute Diagnostics, San Juan, CA). The PCR primers were, (5'), 5'GTGGCTTCTAGatgcatGGGTGGCATC^{3'} (SEQ ID NO:30), and (3'), 5'GAAGGACACctctagaGACAAAATGATGC^{3'} (SEQ ID NO:31), where the capital letters correspond to the HGH sequences and the small letters correspond to the recognition sites for NsiI (5' primer) and XbaI (3' primer).--

Please replace the paragraphs bridging pages 57 and page 58 with the following paragraph.

--Plamid H6E was first made by inserting a 3.7 kb ExoRI-XbaI fragment from the human genomic TTP clone (29) into the plasmid vector pBS+ (Stratagene). This insert contained ~1kb or promoter, the first exon, the intron, the second intron, and 30 bp of 3'-flanking region. For H6E.HGH3', a 597 bp NsiI-XbaI fragment in the 3'-UTR of the human TTP gene that contained five rapid degradation signal sequences was replaced by the entire 110 bp human growth hormone (HGH) 3'-UTR. The PCR primers used to amplify this fragment were (5'), 5'-GTGGCTTCTAGatgcatGGGTGGCATC-3' (SEQ ID NO:36), and (3'), 5'-GAAGGACACctctagaGACAAAATGATGC-3' (SEQ ID NO:37), where the capital letters represent the HGH sequences and the small letters represent the recognition sites for NsiI (5' primer) and XbaI (3' primer).--

Please replace the paragraphs bridging pages 58 and page 59 with the following paragraph.

-- 25. RNA probes were prepared as follows: Plasmid p3'mTNF α , containing the mouse TNF α 3'-UTR (bases 1110-1627 of GenBank accession number X02611) was created by RT-PCR, with the use of total cellular RNA from Raw 264.7 cells treated for 4 hrs with 1 μ g of LPS, as templates for RT. The 5' primer was 5'-CTTTCCgaattcACTGGAGCCTC-3' (SEQ ID NO:32), and the 3' primer was 5'-TAGAtctagaAGCGATCTTTATTTCTCTC-3' (SEQ ID NO:33), with the small letters indicating the restriction sites for EcoRI and XbaI, respectively. The resulting PCR fragment was digested and cloned into the EcoRI and XbaI sites of the vector pSK- (Stratagene). Plasmid pTNF α 1197-1350 contained a 153 bp fragment that included the ARE of the mouse TNF α 3'-UTR (1197-1350 of X02611); this was made using plasmid p3'mTNF α as the template. The 5' primer was 5'-GATAagatctCAGGCCTTCC-3' (SEQ ID NO:34), and the 3' primer was 5'-GCCTtctagaTAAATACATTCATAAGC-3' (SEQ ID NO:35). The resulting PCR product was digested with BglII and XbaI (sites indicated by small letters in the primers) and cloned into the BamHI and XbaI sites of the vector pSK-. Plasmid pTNF α 1281-1350 contained the seven AUUUA motifs of the TNF α ARE (1281-1350 of X02611). This was constructed using similar methods. Correct sequences of these plasmids were confirmed by dideoxy sequencing (Amersham Life Sciences Inc., Arlington Heights, IL). To radiolabel the RNA transcripts with α -³²P-UTP (800 Ci/mmol), plasmid TNF α 1197-1350 was linearized with XbaI and used as the template in the Promega Riboprobe in vitro Transcription System protocol (Promega, Madison, WI). The resulting product was precipitated with ammonium acetate and ethanol.--

Please replace the paragraphs bridging pages 59 and page 60 with the following paragraph.

-- 27. Confluent dishes were washed three times with cysteine-free medium supplemented with 10% FCS. Cells were stimulated for 4 hrs in the same medium with control conditions

(Con), 1 µg/ml LPS or 10 ng/ml TNFα. For the last three hours of the incubation, 200 µCi/ml of 35S-cysteine (NEN Life Sciences, Boston, MA) were added to the cultures. Cells were washed twice with ice-cold PBS, scraped into 10 ml of PBS and pelleted by centrifugation (1000 g for 5 min at 4°). Cells were then resuspended in 600 µl of lysis buffer (50 mM Tris-HCl, pH 7.5; 50 mM NaCl; 3 mM MgCl₂; 5% (v/v) glycerol; 0.5% (v/v) ~~Nonidet P-40~~ NONIDET® P-40 (octylphenolpoly(ethyleneglycolether)) (NP-40); 0.02% (w/v) sodium azide; 5 mM EDTA; 0.1 mM phenylmethylsulfonyl fluoride (PMSF); 20 µg/ml soybean trypsin inhibitor; and 8 µg/ml leupeptin), incubated on ice for 20 min, and lysed by passing 5 times through a 28 gauge needle attached to a 1 ml syringe with no dead space (Becton Dickinson and Company, Franklin Lakes, NJ). Nuclei (pellet after centrifugation at 1000 g for 5 min at 4°C) were washed once in ice-cold wash buffer (10 mM Tris-HCl, pH 7.5; 15 mM KCl; 1.5 mM MgCl₂; 0.5 mM PMSF; and 5% glycerol), centrifuged at 1000 g for 5 min at 4°C, and then resuspended and sonicated in the same volume of lysis buffer used initially to lyse the cells. The cytosolic fraction (supernatant) was clarified by centrifugation at 45,000 g for 30 min at 4°C, using a table-top ultracentrifuge (Beckman TL-100, rotor TLA.45, Beckman Instruments, Inc., Fullerton, CA). This method has been shown to result in clean cytosol-nuclear preparations, when assessed by Western-blotting with an anti-SP1 antibody. Cytosolic extracts matched by trichloroacetic acid-precipitable radioactivity and equivalent volumes of nuclear extracts were pre-cleared with pre-immune rabbit serum (1:100 dilution, 1 hr at 4°C) and protein A-sepharose (1 hr at 4°C), and then incubated overnight at 4°C in the presence of either pre-immune serum (1:100) or a 1:100 dilution of a polyclonal rabbit anti-mouse immune serum (17, 18). Immune complexes were recovered by centrifugation after the addition of protein A-sepharose, washed three times with wash buffer (50 mM Tris-HCl, pH 8.3; 150 mM NaCl; 1 mM EDTA; 0.5% NP-40), resuspended in 100 µl of SDS-sample buffer (28), and subjected to 9% SDS-polyacrylamide gel electrophoresis. Prior to autoradiography, gels were fixed and treated with Autofluor (National Diagnostics, Atlanta, GA).--

Please replace the paragraph on page 65, lines 16-30 with the following paragraph.

-- Plasmid p3'mTNF α containing the mouse TNF α 3'UTR (bp 1110-1627 of GenBank accession number X02611) was constructed by RT-PCR, using RNA from Raw 264.7 cells treated for 4 hrs with 1 μ g/ml of LPS (Sigma, St. Louis, MO) as a template for RT. The 5' primer for PCR amplification was 5'CTTTCCgaattcACTGGAGCCTC^{3'} (SEQ ID NO:32), and the 3' primer was 5'TAGAtctagaAGCGATCTTTATTCTCTC^{3'} (SEQ ID NO:33), where the small letters indicate the restriction sites for EcoRI and XbaI, respectively. The resulting PCR product was digested with these enzymes and cloned into the EcoRI and XbaI sites of the vector pSK- (Stratagene).--

Please replace the paragraph bridging pages 65 and 66 with the following paragraph.

--Plasmid pTNF α 1197-1350, which contained a 153 bp fragment containing the AU-rich element (ARE) of mouse TNF α 3'UTR (bp 1197-1350 of GenBank accession number X02611), was made by PCR using plasmid p3'mTNF α as a template, with a 5' primer, 5'GATAagatctCAGGCCTTCC^{3'} (SEQ ID NO:34), and a 3' primer, 5'GCCTtctagaTAAATACATTCATAAGC^{3'} (SEQ ID NO:35). The resulting PCR product was digested with BglII and XbaI (sites indicated by small letters in the primers) and cloned into the BamHI and XbaI sites of the vector pSK.--

Please replace the paragraph on page 66, lines 4-9 with the following paragraphs.

--Plasmid pTNF α 1197-1300 (bp 1197-1300 of GenBank accession number X02611), containing only one AUUUA motif, was made using the TNF α 3'UTR as template, with the M13-20 primer as the 5' primer, and a 3' primer, 5'CTGAtctagaAGTGCAAATATAAATAGAGG^{3'} (SEQ ID NO:38). The resulting PCR product was digested with EcoRV and XbaI (site indicated by small letters in the 3' primer) and cloned into the corresponding sites of the vector pSK.--

Please replace the paragraph on page 66, lines 11-17 with the following paragraphs.

Plasmid pTNF α 1281-1350 (bp 1281-1350 of GenBank accession number X02611) contained seven AUUUA motifs, five of them being overlapping UUAUUUAUU nanomers. This was constructed using the TNF α 3'UTR as template, with a 5' primer, 5'GACTggatccTCTATTTATATTTGCAC3' (SEQ ID NO:39), and the M13 reverse primer as the 3' primer. The resulting PCR product was digested with BamHI (site indicated by small letters in the 5' primer) and XbaI and cloned into the corresponding sites of the vector pSK.--

Please replace the paragraph bridging pages 67 and 68 with the following paragraph.

--Cell extracts (5-50 μ g protein) were mixed with 1/5 volume of 5X SDS sample buffer (2), boiled for 5 min, then loaded onto 10% SDS-PAGE gels. Western blotting was performed by standard techniques. Membranes were incubated in Tris-buffered saline/0.5% ~~Tween-20~~ TWEEN® 20 (polyoxyethylene sorbitan monolaureate) (TBS/T) with either polyclonal antiserum HA.11 (1:2,500), or a rabbit antiserum to mouse TTP, 2640 (1:100; (38)), or a rabbit antiserum to human TTP, DU88 (1:100; (32)). Incubation of the membranes with second antibody and development were performed as described (6).--

Please replace the paragraph bridging pages 89 and page 90 with the following paragraph.

--Cytosolic extracts were prepared from HEK 293 cells 24 h after the removal of the transfection mixture. The cells were incubated on ice for 20 min in a buffer consisting of 10 mM HEPES (pH 7.6), 3 mM MgCl₂, 40 mM KCl, 5% (v/v) glycerol, 0.5% (v/v) ~~Nonidet-P40~~ NONIDET® P-40, 2 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 8 μ g/ml leupeptin (lysis buffer). Lysis of the cells and maintenance of intact nuclei were carefully monitored by microscopy. The nuclei and cell membrane debris were removed by centrifugation at 16,000 g at 4°C for 15 min. Glycerol was added to the supernatant (cytosolic extract) to 20 % (v/v), and the resulting extract was stored at 70°C--

Please replace the paragraph on page 90, lines 19-22, with the following paragraph.

-- Plasmid pTNF 1309-1332 (A/G) contained five Gs (underlined) replacing the five flanking As of bp 1309-1332 of GenBank accession number X02611 (UUGUUUGUUUGUUUGUUUGUUUUUU) (SEQ ID NO:45) and was constructed as described for pTNF 1309-1332.--

Please replace the paragraph on page 91, lines 16-23, with the following paragraph.

-- Cell extracts (5-50 μ g protein) were mixed with 1/5 volume of 5X SDS sample buffer (26), boiled for 5 min, then loaded onto 12% or 16% SDS-PAGE gels. Western blotting was performed by standard techniques. Membranes were incubated in Tris-buffered saline/0.3% ~~Tween-20~~ TWEEN® 20 (polyoxyethylene sorbitan monolaureate) (TBS/T) with either polyclonal antiserum HA.11 (1:2,500) or an antiserum to U2AF35 (27). Incubation of the membranes with second antibody and development were performed as described (8). For some blots, 125I-protein A (0.2 μ Ci/ml in TBS/T; Amersham, Arlington Heights, IL) was used in place of second antibody.--

Please replace the paragraph on page 101, lines 9-18, with the following paragraph.

--Examination of Fig. 5B reveals that 34 of the 64 amino acids in the TZF domains (53%) have been conserved among all four proteins from species as diverse as human, Xenopus and zebrafish. These include the RYKTEL (SEQ ID NO:26) lead-in sequence for the first zinc finger, the lead-in sequence KYKTEL (SEQ ID NO:40) in the second zinc finger, and several other amino acids in the inter-finger 18 amino acid spacer, including a G residue at position 27, an acidic residue at 30, an L at 31, an H at 37, and a P at 38. Within both zinc fingers, the canonical CCCH residues were conserved. Within the first finger, an E residue was conserved at position 12, a G at 14, a Y at 18, a basic residue at 21, and a QFA at 23-25. Within the second finger, a G residue was conserved at position 52, a Y at 56, an R at 59, an F at 62, and a branched chain amino acid at 63.--

Please replace the "Sequence Listing" with the attached substitute copy of the "Sequence Listing" which includes all previously submitted data with the amendment incorporated therein. Please be advised that the contents of the paper and the computer readable form of the Sequence Listing submitted herewith in the above-identified patent application are the same and include no new matter, as required by 37 C.F.R. 1.821(e), 1.821(f), 1.821(g), 1.825(b), or 1.825(d).

Appendix: Substitute Copy of the "Sequence Listing"